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Ectopic Expression of ζ-Carotene Desaturase Gene from *Oncidium* Gower Ramsey Alters Leaf Morphology and Increases Carotenoid Content in Transgenic Tobacco

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Abstract

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The bright yellow floral color of *Oncidium* Gower Ramsey is due mainly to the accumulation of carotenoids making it an important ornamental orchid in Taiwan. In order to understand the expression pattern of ζ-carotene desaturase gene (*ZDS*), the cDNA encoding *ZDS* was cloned and characterized. The full length of *Oncidium ZDS* cDNA (*OncZDS*) contained 1,986 bp and the 5' and 3' untranslated regions (UTR) were 68 bp and 226 bp, respectively. It had a 1,692 bp open reading frame encoding for a polypeptide of 563 amino acids and shared 72.8–96.2% similarity with other plant species. When analyzing the relative expression level of *OncZDS* in flowers, roots and leaves, the results showed that the expression level of *OncZDS* increased gradually until flowers half opened (stage 5). Furthermore, ectopic expression of *35S::OncZDS* in transgenic tobacco plant resulted in an alteration of leaf morphology and an increase of carotenoid content.

Key words: *Oncidium* Gower Ramsey, ζ-carotene desaturase, Cloning, Gene expression, Ectopic expression.

INTRODUCTION

The flower color of higher plants is mainly composed of three large groups of pigments, betalains, flavonoids and carotenoids. The former two are water-soluble, while carotenoids are lipid-soluble (Grotewold 2006). Anthocyanins belong to the flavonoid group that responsible for the orange, pink, red, purple and blue colors of flowers (He *et al.* 2010). Carotenoids usually accumulate with high concentration in chromoplasts of yellow flowers and display orange, red, bronze and brown colors. Betalains are found in vacuoles in plants of the Cactaceae, Amaranthaceae and Caryophyllales (Christinet 2004) and are a class of yellow, orange, red and purple pigments (To & Wang 2006).

In higher plants, carotenoids are made from C_5 isopentenyl diphosphate (IPP) by methylerythritol phosphate (MEP) pathway in plastids. Four molecules of IPP are converted to geranylgeranyl diphosphate (GGPP) by the action of IPP isomerase (IPI) and GGPP synthase (GGPS). The condensation of two molecules of GGPP by phytoene synthase (PSY) gives rise to 15-cis-phytoene, the first dedi-

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cated compound in the carotenoid pathway. Phytoene is converted into lycopene by the action of two desaturases, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), and displayed in red color. Lycopene is the branching point for subsequent conversion to other compounds (Sandmann 2001; Hieber *et al.* 2006; Giuliano *et al.* 2008). Lycopene cyclase (LYC) catalyzes lycopene to generate α-carotene and β-carotene, respectively. The former was further converted into lutein, astaxanthin and capsorubin, while the latter was converted into zeaxanthin, violaxanthin and neoxanthin, with abscisic acid (ABA) as the final product (Fig. 1) (Cunningham & Gantt 1998; Josse *et al.* 2000; Eisenreich *et al.* 2001; Hirschberg 2001; Moehs *et al.* 2001; Rodríguez-Concepción & Boronat 2002; Giuliano *et al.* 2008).

The genus *Oncidium* is a terrestrial orchid with over 600 species, and native to Mexico, Brazil and Bolivia (Pridgeon *et al.* 1996). The *Oncidium* orchid has become the most important flowers for

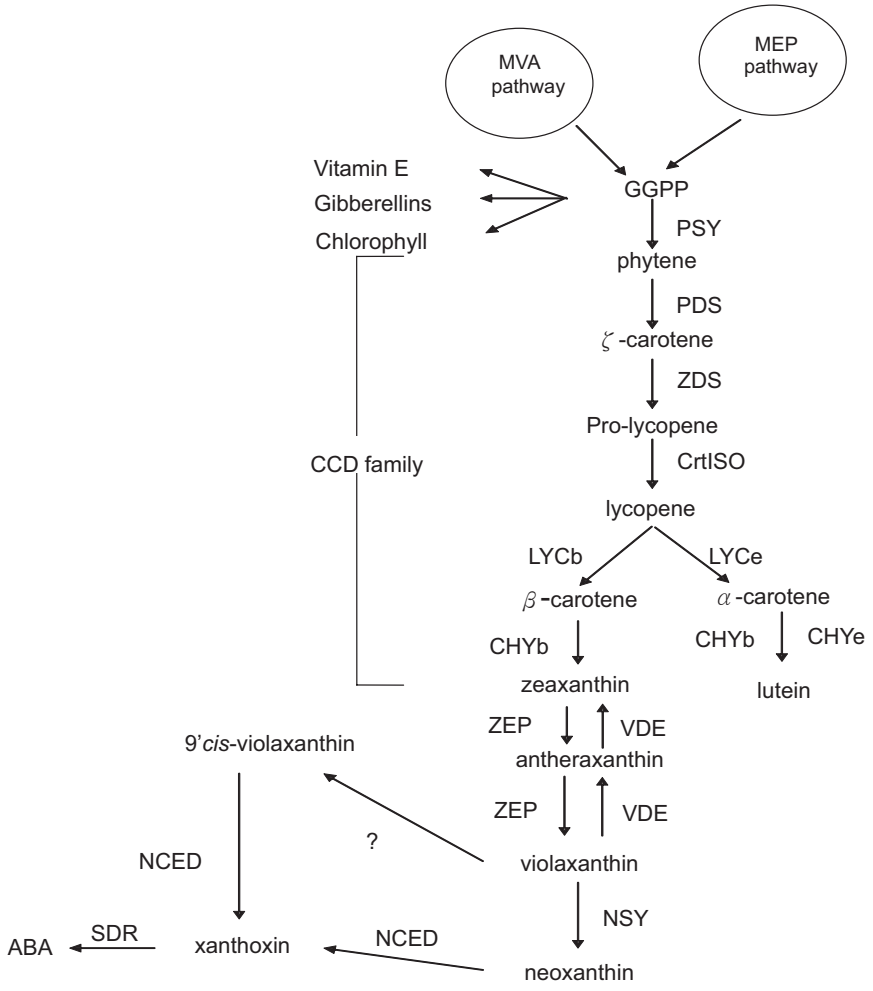

Fig. 1. The pathway of carotenoid biosynthesis in plants. MVA pathway, mevalonic acid pathway; MEP pathway, 2C-methyl-D-erythritol 4-phosphate pathway; GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, f-carotene desaturase; CrtISO, carotenoid isomerase; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; CHYB, carotenoid β-ring hydroxylase; CHYE, carotenoid ε-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; CCD, carotenoid cleavage dioxygenase; NCED, 9-*cis* epoxycarotenoid dioxygenase; ?, putative violaxanthin isomerase; SDR, short-chain dehydrogenase/reductase; ABA, abscisic acid. Figure compiled and summarized from Cunningham & Gantt (1998), Eisenreich *et al.* (2001), Hirschberg (2001), and Rodríguez-Concepción & Boronat (2002).

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commercial production in Singapore, Thailand and other Southeast Asian countries (Chong & Kavaljian 1989). Plants of *Oncidium* Gower Ramsey was introduced to Taiwan in 1986. Because of the suitable location and good adaptability, high quality *Oncidium* cut flowers have been widely accepted by consumers and with market potential. The yellow color of the *Oncidium* flowers is the accumulation of carotenoids in petals and labellum. Those bi-color flowers were a mixture of carotenoids and anthocyanins (Hieber *et al*. 2006). To understand how the genes of carotenoids biosynthesis play a role in different developmental stages and their levels of expression, this study was designed to clone, characterize and examine the expression levels of ζ-carotene desaturase (*ZDS*) gene from *Oncidium* Gower Ramsey in the transgenic tobacco following the developmental sequence. The functional analysis of the gene was also applied.

MATERIALS AND METHODS

Plant materials

The flowering plants of *Oncidium* Gower Ramsey were grown in a greenhouse at Fengshan Tropical Horticulture Experiment Branch, Taiwan Agricultural Research Institute. Flower buds and fully-opened flowers, mature leaves and roots were sampled in the five developmental stages for total RNA extraction and subsequent gene expression analysis (Fig. 2).

RNA extraction and cDNA synthesis

Total RNAs were extracted by using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer's instruction. Quality of the purified RNA was determined by measuring the ratio of absorbance at 260 and 280 nm on a spectrophotometer (U2000, Hitachi Instruments, Tokyo, Japan) and by agarose gel electrophoresis. RNA with ratio of OD260/OD280 ranging from 1.8 to 2.0 was used for cDNA synthesis. The cDNA of *Oncidium ZDS* was PCR (polymerase chain reaction) cloned by using degenerate primers based on conserved amino acid residues of several plant species from NCBI (National Center for Biotechnology Information) database. The primers used in this study are shown in Table 1. The cDNA was synthesized by using SMART™ PCR cDNA Synthesis kit (Clontech, California, USA). The procedure is as follows: 0.5 μg total RNA, 1 μL SMART II primer (10 μM), and 1 μL 3' RACE CDS primer (10 μM) were included in a final volume of 5 μL reaction solution. After incubating at 70℃ for 2 min, 2 μL 5× first-strand buffer, 1 μL DTT (dithiothreitol) mix (20 mM), 1 μL dNTP (deoxy-ribonucleoside triphosphate) mix (10 mM), and 200 units of Superscript II MMLV (Moloney Murine Leukemia Virus) were added to the reaction mixture. The tube was then incubated at 42 ℃ for 1 h. Two μL of first strand cDNAs, 4 μL SMAR II (10 μM) and 5' RACE CDS primers (10 μM, Table 1), 10 μL 10× Advantage 2

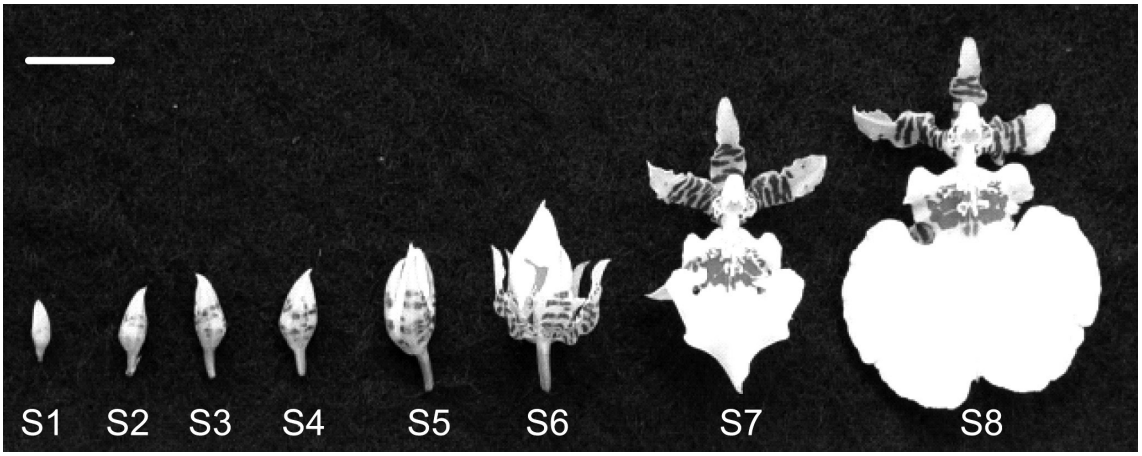

Fig. 2. Morphology of eight flower development stages. Length of flower bud: S1, < 0.5 cm bud; S2, 0.5–0.7 cm bud; S3, 0.7–0.9 cm bud; S4, 0.9–1.0 cm bud; S5, 1.0–1.1 cm bud; S6, 1.1–1.2 cm bud; S7, 1.2–2.5 cm half-open flower; and S8, fully open flower. Bar = 1 cm.

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Table 1. Degenerated and other primer pairs used for *OncZDS* cloning, plasmid construction and gene expression analysis in this study.

Primer name	Sequence of primer
D-ZDS forward	5'-AAYGCNCCNAARGGNYTNTTYCCN-3'[z]
D-ZDS reverse	5'-CARGAYTAYATYGAYTCNATGGAR-3'
G-ZDS forward	5'-TTGACTGTGATAACATCAGTGCTCGGTGTATG-3'
G-ZDS reverse	5'-CAAGATCTGCAAAGCAGGAGAAGTCAGCATCTGGAG-3'
N-ZDS forward	5'-GTATGCTCACCATCTTTGCTCTGTTTGC-3'
N-ZDS reverse	5'-ACAGATTATCAAACATGTCCCATTCTCTCC-3'
E-ZDS forward	5'-GGGCTAAAGTTTTCGATCTGAATCC-3'
E-ZDS reverse	5'-TTCACTATTATACTACAGGGCACACTCAGC-3'
P-ZDS forward	5'-GCG GGA TCC GGGCTAAAGTTTTCGATCTGAATCC-3'[y]
P-ZDS reverse	5'-GCG GAG CTC TTCACTATTATACTACAGGGCACACTCAG-3'[x]
SMART II	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
3' RACE CDS	5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
5' RACE CDS	5'-$(T)_{25}N_{-1}N$-3'
UPM-long	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAG AGT-3'
UPM-short	5'-CTAATACGACTCACTATAGGGC-3'
NUP	5'-AAGCAGTGGTATCAACGCAGAGT-3'
actin forward	5'-AGTTGAACCTCCACTGAGCAC-3'
actin reverse	5'-GGGCACCTGAACCGCTCGGCTCC-3'
353-F	5'-AAGATACAGTCTCAGAAGACCAAAGG-3'

[z] Y = C, T; W = A, T; D = A, G, T; R = A, G; V = A, C, G; H = A, C, T; M = A, C, N_{-1} = A, G or C; and N = A, C, G or T.
[y] Underlined sequences indicate the cutting site of restriction enzyme *Bam*HI.
[x] Underlined sequences indicate the cutting site of restriction enzyme *Sac*I.

PCR Buffer, 2 μL dNTP mix (10 mM) and 2 μL Advantage 2 PCR Polymerase Mix (Clontech, Mountain View, California, USA) were mixed together and proceeded for long distance PCR to amplify double-strand cDNA. The PCR reaction was programmed as follows: 1 initial denaturing cycle at 95℃ for 1 min, 24 cycles of 5 s denaturing at 95℃, 5 s annealing at 65℃, 6 min extension at 68℃ (ABI Gene Amp PCR system 2700). The quality of the double-strand cDNAs was checked by 1% agarose gel electrophoresis.

Rapid amplification of cDNA ends (RACE)

SMART™ RACE cDNA Amplification kit (Clontech, Mountain View, California, USA) was used for full length *OncZDS* amplification. Brief steps are as follows: 3 μL total RNA, 1 μL 5'-CDS primer, and 1 μL SMART II A oligo were mixed in a 0.2 mL tube, and heated for 2 min at 70℃, and cooled for 2 min on ice. Then 2 μL 5× first-strand buffer, 1 μL 20 mM DDT, 1 μL 10 mM dNTP mix, and 1 μL Power Script Reverse MMLV transcriptase (Roche Molecular Systems, Pleasanton, California, USA.) were mixed and incubated at 42℃ for 1.5 h, and heated at 70℃ for 7 min to end the reaction, and stored at -20℃. Three μL of total RNA, 1 μL 5'-CDS primer, and 1 μL ddH_2O were mixed as described above to synthesize 3'-RACE-Ready cDNA. PCR cycles were programmed as follows: 5 cycles of 10 s denaturation at 94℃, 3 min annealing and extension at 72℃. Five cycles of 10 s denaturation at 94℃, 15 s annealing at 71℃, 3 min extension at 72℃, and then 20 cycles of 10 s denaturation at 94℃, 15 s annealing at 70℃, 3 min extension at 72℃. PCR products were stored at 4℃ until use.

PCR product purification and cloning

PCR product was purified by using QIAEX®II Gel Extraction kit (Qiagen, Valencia, California, USA). DNA bands were cut off the agarose gel after

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electrophoresis, and placed into a 1.5 mL tube to which 300 μL Buffer QX1 was added per 0.1 g gel block. After adding 10 μL QIAEXII., the mixture was heated at 50℃ for 10 min, centrifuged for 30 s at 13,000 rpm (16,110× *g*), and the supernatant discarded and the precipitate washed by using 500 μL Buffer QX1 twice. The DNA pellet was redissolved with 20 μL ddH_2O. The purified PCR fragment was ligated into pGEM-T Easy vector by using T4 DNA ligase (Promega, Madison, Wisconsin, USA), and transformed into competent cells of JM109 at 42℃ by heat shock, and streaked on LB/ampicillin (100 μg mL^{-1}) medium containing X-gal (40 mg mL^{-1}) and IPTG (50 mg mL^{-1}) for 18 to 20 h at 37℃. Those white colonies were individually picked and analyzed for insert fragment check by PCR.

Bioinformatics analysis

The analysis of the deduced amino acid sequence was performed by using Omiga 2.0 software (Oxford Molecular, Cambridge, United Kingdom). Amino acid sequence comparison with homologs from other plants was conducted by using BLAST of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was performed by using MegAlign program of DNAstar software (DNAstar, Madison, USA).

Semiquantitative RT-PCR analysis

Total RNA was extracted from flower buds, open flowers, leaves and roots by the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). Single strand cDNA was synthesized by using the SMART™ PCR cDNA Synthesis kit following the instruction of the manufacturer (Clontech, Mountain View, California, USA). The specific primer pairs G-PSY forward, G-PSY reverse and *actin* primer pairs (Table 1) were used for PCR amplification. The housekeeping gene *actin* was designed based on the sequence of *Populas trichocarba* (EF418792) using the Lasergene Primer Select (DNAStar, Madison WI, USA) software. PCR cycles were programmed as follows: 1 cycle of 2 min denaturation at 94℃. Five cycles of 10 s denaturation at 94℃, 10 s annealing at 60℃, 40 s extension at 72℃, and then 27 cycles of 10 s denaturation at 94℃, 10 s annealing at 55℃, and 40 s at 72℃. PCR products were stored at 4℃ until use. The PCR products of *OncPSY* and *actin* were separated on a 2% agarose gel and analyzed by Alpha Imager software (Clontech, Mountain View, California, USA). Data were analyzed and chart generated by using Sigma Plot 9.0 software (Systat software, San Jose, California, USA.).

Plasmid construction

The *OncZDS* coding sequence was amplified from *Oncidium* Gower Ramsey labellum cDNA using P-ZDS primer pairs (Table 1). After the cleavage with *Bam*HI and *Sac*I, the *OncZDS* fragment was ligated into *Bam*HI and *Sac*I restriction site of the binary vector pBI121 and the resulting vector designated pBIZDS. The vector pBIZDS was re-cleaved with *Hind*III and *Eco*RI to generate a fragment containing 35S promoter, *OncZDS* gene and NOS terminator and was then ligated into another binary vector pCAMBIA 1305.1, renamed as pOncZDS to provide GUS gene for identification of transgenic plant.

Plant transformation and regeneration

Nicotiana tabacum cv. Xanthi NC was transformed with the construct pOncZDS by *Agrobacterium tumefaciens*. The transformation was performed as follows: tobacco seeds were surfaced sterilized and sown in Murashige & Skoog (1962) medium for germination. Leaves were cut into 0.5 cm × 0.5 cm disks from 4 wk old seedlings and co-cultured with *Agrobacterium tumefaciens* LBA4404/pOncZDS at 26℃ for 3 d in the dark then washed with sterile water and transferred onto MS medium containing 0.5 mg L^{-1} benzylaminopurine, 50 mg L^{-1} hygromycin and 300 mg L^{-1} cefotaxime. Hygromycin resistant adventitious shoots were cut off and transferred onto fresh selection medium without hormone. The established plantlets were potted, grown in the greenhouse and positive transformant identified by GUS stain, PCR and southern hybridization.

Pigment isolation and quantification

Fifteen mL of hexane/acetone/ethanol (2:1:1, v/v/v) was added to 0.3–0.6 g of fresh *Oncidium* tissues (flower buds, leaves and roots) and mixed thoroughly until the tissue was bleached. The organic solvent phase was separated by the addition of 2 mL water and the hexane fraction dried in a stream of nitrogen gas at room temperature and resuspended in acetone. Total carotenoids and chlorophylls were determined by spectrophotometrically method (Arnon *et al.* 1954).

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RESULTS

Cloning ζ-carotene desaturase gene from *Oncidium* Gower Ramsey

To isolate the *ZDS* gene from *Oncidium* Gower Ramsey, a strategy that combined RT-PCR and RACE was used. A cDNA fragment was amplified by RT-PCR using total RNA from labellum as a template. Comparison of deduced amino acid sequence with several ZDS led to the confirmation of partial sequence of *Oncidium ZDS*. The full-length *ZDS* cDNA sequence from *Oncidium* Gower Ramsey (*OncZDS*) was obtained by using RACE. The *OncZDS* contains 1,986 nucleotides, with 68 bp and 226 bp of 5' and 3' untranslated regions (UTR), respectively. *OncZDS* encodes a protein with 563 amino acids, which shows high sequence similarity to the peptide sequence of orchid (*Oncidium* Gower Ramsey, ACP27625, 99.5% identity) and tomato (*Solanum lycopersicum*, ABD67160.1, 75% identity).

Phylogenic analysis of *OncZDS*

Comparisons of *OncZDS* with ZDS of 24 other plant species were carried out by BLASTp method (Altschul *et al*. 1997). The results showed all of 25 ZDS sequences perfectly separated into two groups. One was dicotyledonous group, including citrus (*Citrus sinensis*, CAC85667.1; *Citrus unshiu*, BAB68552.1; *Citrus maxima*, ACE79169.1; *Citrus x paradise*, AAK51557.1), black cottonwood (*Populus trichocarpa*, XP_002319628.1), barbados nut (*Jatropha curcas*, ACT87979.1), apple (*Malus x domestica*, AAQ04225.1), strawberry (*Fragaria x ananassa*, ACR61394.1), papaya (*Carica papaya*, ACO40527.1), arabidopsis (*Arabidopsis thaliana*, AAM63349.1), turnip rape (*Brassica rapa* subsp. *pekinensis*, ACM68701.1), grape (*Vitis vinifera*, XP_002277348.1), great yellow gentian (*Gentiana lutea*, BAA88843.1), tomato (*Solanum lycopersicum*, ABD67160.1), chili pepper (*Capsicum annuum*, 2121278A), wild carrot (*Daucus carota* subsp. *sativus*, ABB52070.1), sunflower (*Helianthus annuus*, CAD55814.2), chrysanthemum (*Chrysanthemum x morifolium*, BAE79555.1) and cream narcissus (*Narcissus tazetta* var. *chinensis*, ABX45112.1), the other group, monocotyledonous includes corn (*Zea mays*, ACG46735.1),sorghum (*Sorghum bicolor*, AAX56323.1), bread wheat (*Triticum aestivum*, ACI04664.1), rice (*Oryza sativa* Japonica Group, NP_001059145.1) and yellow *Oncidium* (*Oncidium* 'Gower Ramsey', ACP27625.1) (Fig. 3, Table 2).

Expression of *OncZDS* in *Oncidium* tissues

To explore the expression patterns for *OncZDS* in different tissues of *Oncidium* Gower Ramsey, semi-quantitative RT-PCR analysis was used. As the expression levels of both *OncZDS* and *actin* genes within 32–38 PCR cycles were visually detectable in agarose gel, 36 cycles were chosen for semi-quantitative determination of *OncZDS* in this study. The transcript level of *OncZDS* was detected in roots, leaves, and different flower bud stages (S1–S8). The transcript level of early steps of the carotenoid biosynthesis pathway gene *OncZDS* was high in vegetative tissues and flower, and lower in root. During flower development, *OncZDS* expression increased as flower developed until stage 5 and 6 (Fig. 4). The *OncZDS* expression showed a downward trend, while newly open flowers (with labellum visible, stage 6 and 7) increased until flower fully open (stage 8). Meanwhile, total chlorophyll content was highest at stage 1 buds and decreased steadily as flowers matured; while total carotenoids content increased with flower development until fully open (data not shown).

Construction of plant transformation vector

Plasmid pBI121 was used for subcloning to retain the GUS gene as a reporter. Restriction sites were generated by E-ZDS forward and E-ZDS reverse primer pairs by PCR, generating a 1,849 bp fragment which contained *Bam*HI and *Sac*I cutting sites. Both two restriction enzymes help the directional cloning of *OncZDS* into plasmid pBI121 and the generation of new plasmid named pBIZDS. The intermediate vector pBIZDS was cut by *Hind*III and *Eco*RI, and 2,720 bp fragment containing 35S promoter region (871 bp), *OncZDS* gene (1,849 bp) and NOS terminator region (267 bp) were obtained. The fragment was subcloned into pCAMBA1305.1 vector by restriction digestion (*Hind*III and *Eco*RI) and the ligation product generated new binary vector named pOncZDS. To confirm the correctness of the plasmid construction, the results from PCR by 35S and NOS primers proved the *OncZDS* insertion in expected location. Meanwhile, 35S-F and E-ZDS-re-

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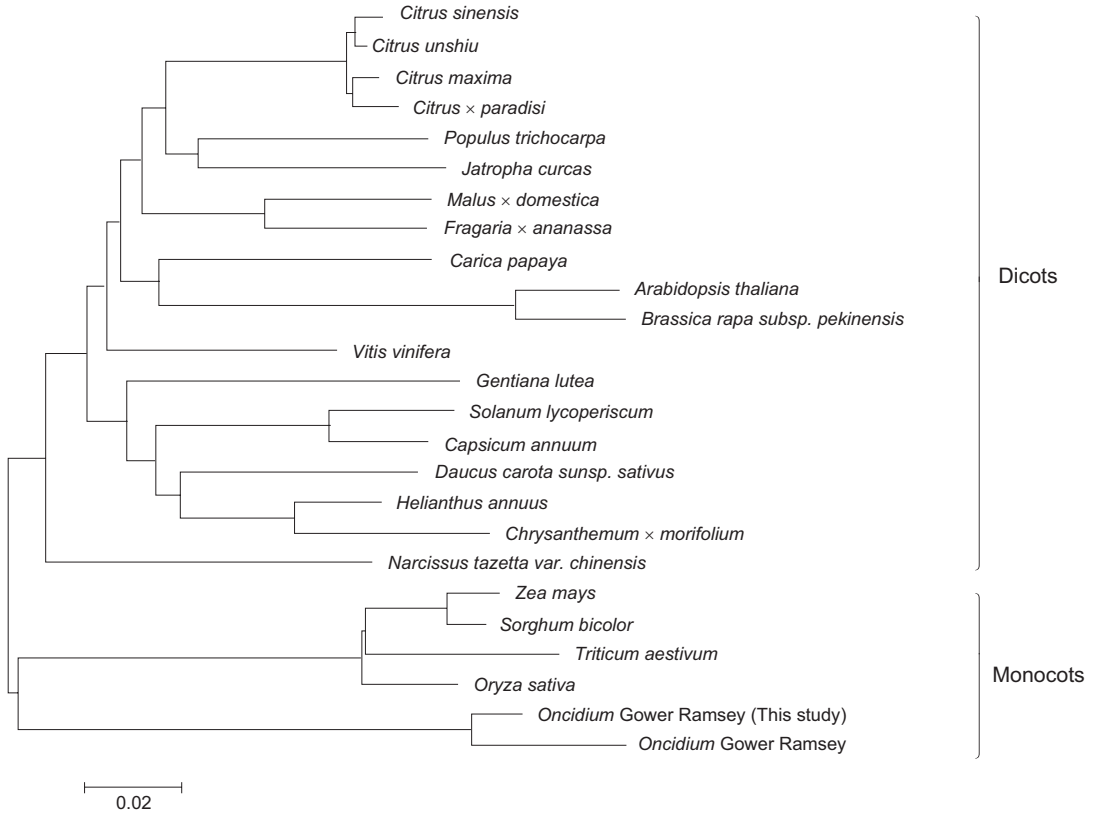

Fig. 3. Phylogenetic tree of *Onc*ZDS with other plants on amino acid sequence of full length protein by BLAST programs. Scale bar represents 0.02 expected amino acid residue substitutions per site. *Citrus sinensis*, CAC85667.1; *Citrus unshiu*, BAB68552.1; *Citrus maxima*, ACE79169.1; *Citrus* × *paradise*, AAK51557.1; *Populus trichocarpa*, XP_002319628.1; *Jatropha curcas*, ACT87979.1; *Vitis vinifera*, XP_002277348.1; *Malus* × *domestica*, AAQ04225.1; *Fragaria* × *ananassa*, ACR61394.1; *Carica papaya*, ACO40527.1; *Gentiana lutea*, BAA88843.1; *Solanum lycopersicum*, ABD67160.1; *Capsicum annuum*, 2121278A; *Daucus carota* subsp. Sativus, ABB52070.1; *Helianthus annuus*, CAD55814.2; *Chrysanthemum* × *morifolium*, BAE79555.1; *Narcissus tazetta var*. chinensis, ABX45112.1; *Arabidopsis thaliana*, AAM63349.1; *Brassica rapa* subsp. Pekinensis, ACM68701.1; *Triticum aestivum*, ACI04664.1; *Oryza sativa* Japonica Group, NP_001059145.1; *Sorghum bicolor*, AAX56323.1; *Zea mays*, ACG46735.1; *Oncidium* Gower Ramsey, this study; and *Oncidium* Gower Ramsey, ACP27625.1.

verse primers gave rise to a 2,113 bp DNA fragment; 35S-F and NOS-3 primers with a 2,380 bp fragment. Southern hybridization also confirmed the results of PCR check, indicating the binary vector pOncZDS was as good as designed (Fig. 5).

Ectopic overexpression of *35S:: OncZDS* alters leaf morphology and increases carotenoid content

The p*OncZDS* containing coding sequence of *OncZDS* was expressed in sense orientation under control of a duplicated 35S RNA promoter in transgenic tobacco. Transgenic plants obtained by *Agrobacterium tumefaciens* mediated transformation of leaf discs (Fig. 6A) were transplanted and moved to the greenhouse; after 6 to 7 wk, the contents of carotenoids and chlorophyll in the plants were determined by a spectrophotometer. The transgenic tobacco showed insignificant change of flower color as compared to the wildtype (Fig. 6B). The leaves of transgenic tobacco showed signs of shrinkage and bifurcation (Fig. 6C and Fig. 6D) with a smaller size (Fig. 6E and Fig. 6F). The transgenic tobaccos showed increased carotenoids and chlorophyll contents up to 235.4 ± 2.0, and 158.9 ± 3.9 μg g^{-1} FW, respectively. In average, transgenic tobacco plants increased

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Table 2. Amino acid sequence similarity (%) of OncZDS compared to the homologous genes of some other plant species.

	1[z]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	100																								
2	99.2	100																							
3	98.5	98.7	100																						
4	98.5	98.7	98.3	100																					
5	85.7	86.1	85.9	85.7	100																				
6	84.6	85.2	84.6	84.2	86.9	100																			
7	84.6	85.1	84.9	84.6	82.6	82.1	100																		
8	85.9	86.2	86.2	85.9	84.4	82.9	84.6	100																	
9	86.1	86.6	86.1	85.9	83.1	82.6	83.4	91.7	100																
10	86.6	86.9	86.4	86.1	85.1	84.4	84.1	83.4	83.9	100															
11	80.3	80.8	80.4	80.1	78.6	78.8	81.3	79.6	79.4	78.6	100														
12	80.6	81.1	80.8	80.4	79.6	78.9	80.1	79.6	79.9	78.6	81.9	100													
13	81.4	81.9	81.6	81.3	79.3	79.3	80.3	80.4	80.8	79.8	81.3	94.2	100												
14	82.6	83.1	82.8	82.4	81.4	80.6	82.4	81.1	81.4	81.6	81.6	83.3	82.3	100											
15	81.6	82.1	81.6	81.6	80.6	80.3	81.6	81.3	80.3	80.4	82.6	84.6	84.4	86.1	100										
16	80.9	81.4	80.9	80.6	79.3	78.1	79.9	80.1	78.9	78.6	80.1	82.6	83.1	84.2	90.9	100									
17	83.4	83.7	83.1	82.8	80.8	80.8	82.4	82.3	81.4	81.4	78.6	78.8	78.9	80.9	80.4	79.1	100								
18	81.8	82.1	81.9	81.4	79.8	78.6	82.1	80.4	80.9	83.1	75.8	76.6	76.1	78.8	77.3	77.1	79.4	100							
19	82.6	82.9	82.8	82.3	80.1	79.3	80.4	80.4	80.4	82.1	75.3	76.3	75.8	78.4	77.1	76.5	79.4	94.2	100						
20	77.4	77.6	77.6	77.4	75.3	74.0	78.1	78.9	78.9	77.3	75.1	75.6	76.3	75.0	75.8	75.0	77.9	76.1	75.6	100					
21	78.6	78.9	78.8	78.6	76.5	74.6	78.9	79.8	79.3	77.4	77.1	77.4	77.4	76.5	76.6	75.5	77.9	76.6	76.3	89.9	100				
22	78.1	78.3	78.4	77.9	75.8	74.1	78.6	79.3	79.8	77.1	76.5	77.8	78.6	76.1	77.3	75.6	77.4	76.5	76.3	90.0	91.0	100			
23	77.9	78.1	78.3	77.8	75.8	73.8	78.4	79.1	79.9	76.8	76.0	77.6	77.9	76.1	76.9	75.5	76.9	76.8	76.6	89.1	70.7	97.0	100		
24	77.9	78.1	77.8	77.3	74.8	74.1	76.9	77.8	77.8	77.3	74.8	73.6	74.3	74.1	73.8	72.8	78.4	75.6	76.0	75.6	75.6	75.8	75.5	100	
25	79.6	79.8	79.4	78.9	76.3	76.0	78.6	79.3	79.3	79.3	76.6	75.0	75.6	76.0	75.5	74.5	80.6	77.6	77.9	77.3	77.3	77.4	77.1	96.2	100

[z] 1: *Citrus sinensis*, CAC85667.1; 2: *Citrus unshiu*, BAB68552.1; 3: *Citrus maxima*, ACE79169.1; 4: *Citrus x paradise*, AAK51557.1; 5: *Populus trichocarpa*, XP_002319628.1; 6: *Jatropha curcas*, ACT87979.1; 7: *Vitis vinifera*, XP_002277348.1; 8: *Malus x domestica*, AAQ04225.1; 9: *Fragaria x ananassa*, ACR61394.1; 10: *Carica papaya*, ACO40527.1; 11: *Gentiana lutea*, BAA88843.1; 12: *Solanum lycopersicum*, ABD67160.1; 13: *Capsicum annuum*, 2121278A; 14: *Daucus carota* subsp. Sativus, ABB52070.1; 15: *Helianthus annuus*, CAD55814.2; 16: *Chrysanthemum x morifolium*, BAE79555.1; 17: *Narcissus tazetta var*. chinensis, ABX45112.1; 18: *Arabidopsis thaliana*, AAM63349.1; 19: *Brassica rapa* subsp. Pekinensis, ACM68701.1; 20: *Triticum aestivum*, ACI04664.1; 21: *Oryza sativa* Japonica Group, NP_001059145.1; 22: *Sorghum bicolor*, AAX56323.1; 23: *Zea mays*, ACG46735.1; 24: *Oncidium* Gower Ramsey, this study; and 25: *Oncidium* Gower Ramsey, ACP27625.1.

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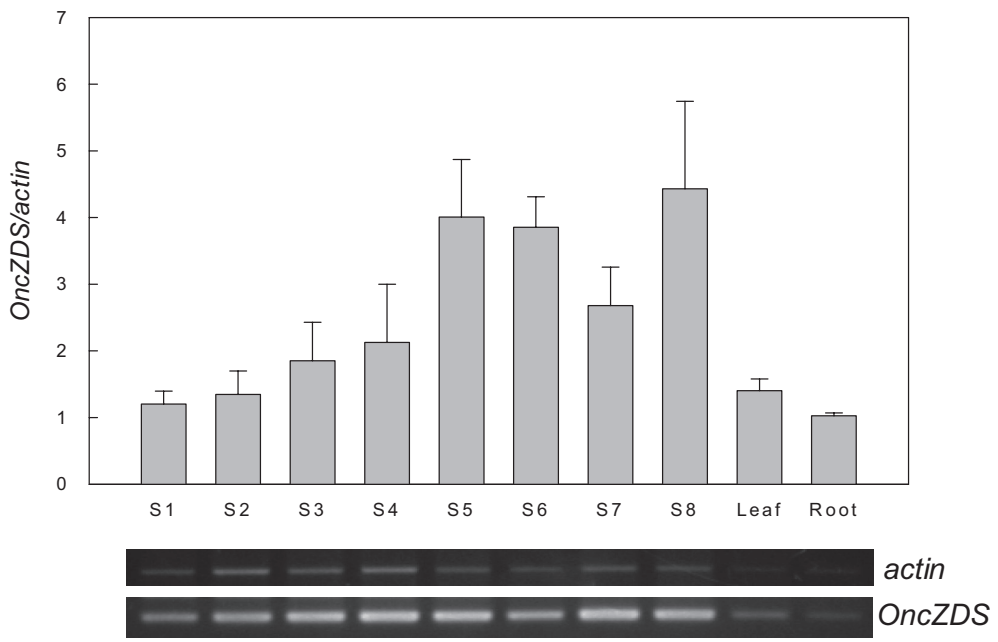

Fig. 4. Relative expression level (%) of ZDS transcripts estimated by RT-PCR. The amplification of *OncZDS* and *actin* transcripts were performed using 5 μg of total RNA isolated from different stages of flower, leaves and roots. The PCR products were resolved on a TAE 2.0% agarose gel. The relative expression level of the *ZDS* transcripts, expressed as a percentage with respect to the *actin* level, was estimated by measuring the intensity of ethidium bromide fluorescence of the PCR products resolved by gel electrophoresis. Error bar is the standard error of mean (n = 3). S1 to S8, different floral stages as shown in Fig. 2.

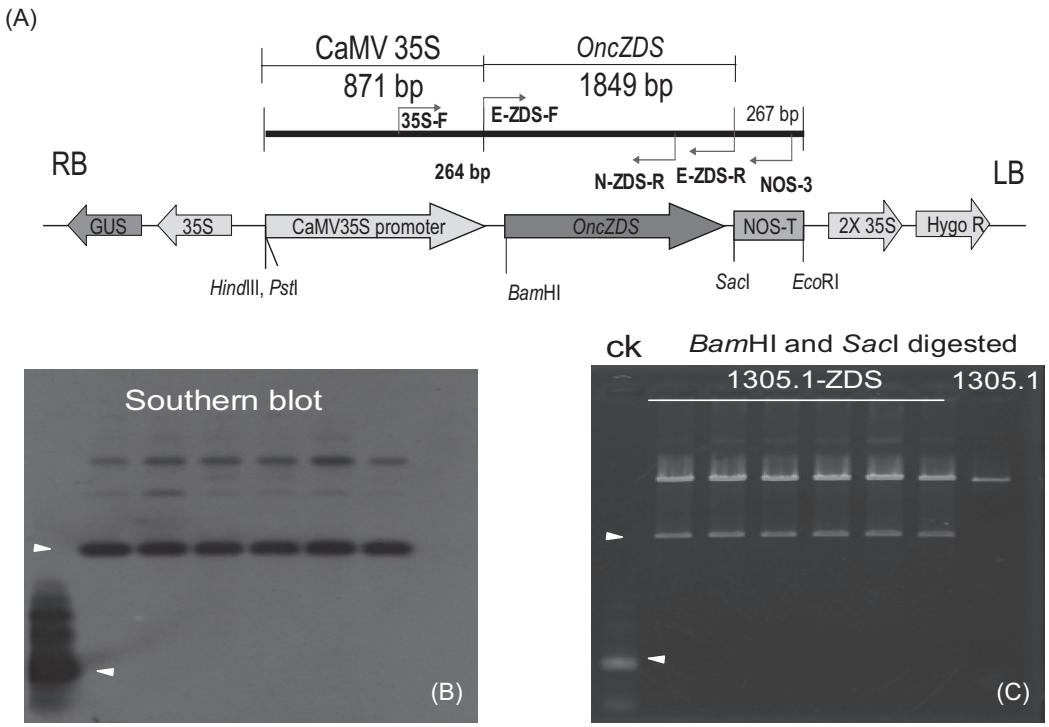

Fig. 5. Construction of p*OncZDS*. (A) Generation of a chimeric construct; p*OncZDS* containing *OncZDS* coding region; (B) PCR confirmed the construct of plasmid; and (C) Southern hybridization confirmation.

13% chlorophyll and 10% carotenoids and the ratio of chlorophyll to carotenoids was also increased (Table 3).

DISCUSSION

In recent years, the regulation of carotenoids in flowers has been widely studied (Naik *et al.* 2003; Giuliano *et al.* 2008; Zhu *et al.* 2010). To further understand the control mechanism of the *Oncidium* floral pigmentation, this study used rapid amplification of cDNA ends to clone the gene of ζ-carotene desaturase (ZDS), which affects carotenoids biosynthesis of flowers. Its amino acid sequence is highly conserved, but with some variations in N-terminal and C-terminal parts of the protein, as compared to other plant ZDS. Phylogenetic analysis just perfectly separated ZDS in all 25 plants, including Oncidium, into two groups of monocots or dicots.

OncZDS expression is tissue-specific and increased by flower expansion levels. In great yellow gentian, *GtZDS* (*Gentiana lutea*) transcript was detected in pistils, stamens, sepals and leaves, and especially in petals, but almost none in the stem (Zhu *et al.* 2002). In *Arabidopsis*, *AtZDS* expressed mostly in the leaves (Dong *et al.* 2007). In chrysanthemum (*Chrysanthemum morifolium*), *CmZDS* was detectable in white, yellow or orange flowers but higher in yellow than white one (Kishimoto & Ohmiya 2006). Meanwhile, ZDS was a key enzyme controlling the carotenoids biosynthesis when flowers unfolded, with its expression increased gradually (Kishimoto & Ohmiya 2006; Chiou *et al.* 2010; Zhu *et al.* 2010).

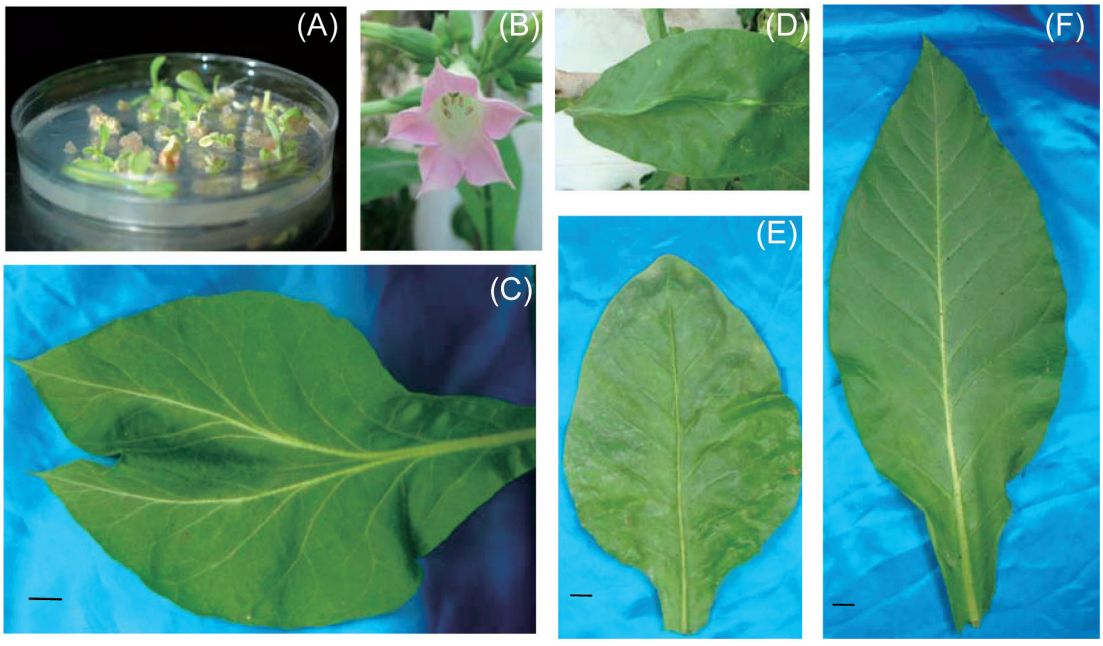

Fig. 6. Ectopic expression of *35S::OncZDS* in tobacco causes severe phenotypic effects. (A) hygromycin was used for selection marker and untransformed tobacco shown etiolating; (B) flower color was insignificant change compared with wile type tobacco; (C) transgenic tobacco lines showed bifurcation leaves; (D) transgenic tobacco showed shrinkage vein; (E) overexpression *35S::Onc*ZDS reduced leaf size; and (F) wild type tobacco leaf. Bar = 1 cm.

Table 3. Chlorophylls and carotenoids of selected transgenic tobacco plants expressing *OncZDS*.

Line	Chlorophyll a (μg g^{-1} FW)	Chlorophyll b (μg g^{-1} FW)	Total chlorophylls (μg g^{-1} FW)	Total carotenoids (μg g^{-1} FW)	Chlorophylls to carotenoids ratio
35S::OncZDS	122.3 ± 3.0[z]	36.1 ± 0.9	158.9 ± 3.9	235.4 ± 2.0	0.68
Control	113.2 ± 2.6	26.7 ± 0.2	140.0 ± 1.5	214.6 ± 1.8	0.65

[z] Mean ± standard error (n = 3).

When ZDS gene was inhibited, not only the flower color but also plant phenotype affected (Qin *et al.* 2007). For example, mutation of *Arabidopsis* ZDS gene caused the appearance of plant in light green color and displayed ABA deficiency symptoms and a downstream biosynthetic pathway of carotenoids (Hirayama *et al.* 1994). Mutation in ZDS of arabidopsis indirectly increased photo-oxidation, leading to increased reactive oxygen species (ROS) content and a bleaching effect. Serious ZDS mutations affect the original physiological function and easy to produce lethal seedlings (Dong *et al.* 2007). Results suggest that the function of ZDS is not just for flower color but is also important for plant growth and development.

Despite insignificant flower color change in *35S::OncZDS* transgenic tobacco, their carotenoids and chlorophylls were increased. Such a result is similar to overexpressing *GlZds* gene of *Gentiana lutea* in transgenic tobacco, with 91% increase of β-carotene in leaves and 49% increase in flowers (Jing *et al*. 2009). Meanwhile, the phenotype of *35S::OncZDS* transgenic tobacco with unusual reduced size, shrinkage and bifurcation of leaves were similar to overexpression of *OncPSY* (Lee 2012).

Alteration of flower color by genetic engineering to modify the flavonoid biosynthetic pathways has already been achieved (Forkmann & Martens 2001). Similar achievements have also been made for carotenoid pigments. When suppressing carotenoids cleavage dioxygenase 4a (CmCCD4a) of white chrysanthemum by RNAi, transgenic plants show yellowish color (Ohmiya *et al.* 2006). Expression antisense CRTL-b2 (LYCb2) mRNA would make yellow potato showing orange petals (Ronen *et al.* 2000). Overexpression tree tobacco, *Ng*CRTO (*Nicotiana glauca*, β-carotene ketolase) in transgenic cyanobacteria, *Synechocystis* sp. PCC 6803 would make yellow flower change into orange (Zhu *et al.* 2010).

In this study, we have cloned full length *ZDS* cDNA from *Oncidium* orchid. Ectopic expression *35S::OncZDS* in transgenic tobaccos caused changes in leaf phenotype and the increase of carotenoids and chlorophylls. Interfering the genes of carotenoid biosynthesis would not only affect color but also phenotype, sometimes even caused a seedling lethal (Hirayama *et al.* 1994; Dong *et al.* 2007). The results from this work and others indicated the importance of ZDS gene function in regulating growth and development of plants. In the future, we will try to screen the promoter for petal specific expression in order to reduce the negative effects of genes on plant physiological processes. Attempts will also be made to increase the diversity of *Oncidium* orchid flower color by genetic manipulation for improving market needs of *Oncidium*, in cut flowers or potted flower plants.

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異體表達文心蘭 (南西) ζ-Carotene Desaturase 致使轉基因煙草胡蘿蔔素含量增加及葉型改變

李文立 [1] 陳福旗 [2,*]

摘要

李文立、陳福旗。2012。異體表達文心蘭 (南西) ζ-Carotene Desaturase 致使轉基因煙草胡蘿蔔素含量增加及葉型改變。台灣農業研究 62(2):93–105。

聚積胡蘿蔔素，具有亮黃色花朵的文心蘭 (南西) 是台灣重要的蘭花。為了瞭解胡蘿蔔素生合成中 ζ- 胡蘿蔔素脫氫酶 (ζ-carotene desaturase; *ZDS*) 的表達方式，我們選殖並分析了文心蘭 (南西) 的 ζ- 胡蘿蔔素脫氫酶。此關鍵酵素，*OncZDS* 具有 1,986 bp 核苷酸，5’ 和 3’ 未轉錄區 (untranslated regions; UTR) 分別是 68 與 226 個核苷酸。*OncZDS* 含有一個長 1,692 bp 的解讀框架 (open reading frame)，可以解碼成 563 個氨基酸的多肽 (polypeptide)，此多肽與其他植物具有 72.8–96.2% 的相似度。*OncZDS* 的相對表現量分析顯示，當花朵開放時，此基因的表現量逐漸增加至花朵半開時 (第 5 時期)。而異體表達此基因會造成轉基因煙草的葉形改變，並增加其總胡蘿蔔素的含量。

關鍵詞：文心蘭 (南西)、ζ- 胡蘿蔔素脫氫酶、選殖、基因表達、過量表達。

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